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(54) Title: ADAPTER MOLECULES FOR TARGETING VIRAL PARTICLES TO CELLS

(57) Abstract

This invention relates to molecular adapter molecules for targeting viral particles, in particular retroviral particles, to cells. The adapter molecules comprise a first amino acid sequence having binding affinity for a cell surface molecule and a second amino acid sequence having binding affinity for a viral surface molecule. The adapter molecules are useful in gene therapy, for targeting retroviral delivery vectors to specific cell types.

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ADAPTER MOLECULES FOR TARGETING VIRAL PARTICLES TO CELLS

This invention relates to adapter molecules which facilitate the binding of virus particles to cell surface molecules. The invention also relates to nucleic acids encoding the adapter molecules, to cell lines expressing the adapter molecules and to the use of the adapter molecules in gene therapy.

INTRODUCTION

A common goal of gene therapy is to be able to deliver genes *in vivo* rather than using the cumbersome *ex vivo* strategies most often used to date. In developing technologies for delivery *in vivo* it would be a significant advantage if it was possible to deliver genes selectively by targeting the delivery system to specific cell types. This invention describes a new strategy for achieving targeted gene delivery using retroviral vectors.

Some success with targeting retroviral vectors has been achieved recently. Current retroviral vectors, used for human applications, are promiscuous in that they carry the amphotropic envelope protein that permits infection of a wide range of cell types from diverse species. This is in contrast to the other main type of retroviral vector that is used in the laboratory which carries the ecotropic envelope which only permits infection of rodent cells. In an attempt to achieve efficient and selective gene transfer *in vivo*, considerable effort is now being directed towards producing retroviral vectors that are targeted to specific human cell types. The main approach has been to construct chimaeric envelope proteins containing ligands such as EGF (Han et al., 1995 PNAS 92, 9747; Cosset et al., 1995 J.Virol 69, 6314), EPO (Kasahara et al., 1994 Science 266, 1375), RGD containing peptides (Valsesia-Wittman et al., 1994 J.Virol 68, 4609), antibody fragments (Russell et al., 1993 Nucl. Acids R s. 21, 1081) or single chain antibodies (Tearina and Domburg., 1995 J.Virol 69, 2659,

Somia et al., 1995 PNAS 92, 7570). In addition chemical modifications of envelope proteins have been used to try to target to liver cells (Neda et al., 1991 J. Biol. Chem. 266, 14143) and biotin-streptavidin bridges have been used to link virus and host cell (Roux et al., 1989 PNAS 86, 9079). In many cases specific binding of these 'targeted' retroviruses to the appropriate cell has been demonstrated but in all cases the efficiency of gene transfer was significantly lower than can be obtained with the amphotropic envelope. Furthermore, all chimaeric envelopes require either co-expression of the unmodified envelope protein or deglycosylation of the virus or treatment of the cells with endosome modifying agents (e.g. chloroquine) to effect any gene transfer at all. Numerous techniques in this area have been published and none of them teach a method of targeting retroviruses to specific cell types at high efficiency.

The common feature of all of these strategies for targeting retroviral vectors is that the SU component of the envelope proteins has been modified in some way to direct it to a specific cell surface molecule. It is conceivable that this type of envelope modification will never lead to efficient targeted gene transfer because the structure and function of the envelope proteins are exquisitely sensitive to change.

There is therefore a need for improved ways of delivering genetic material from retroviral vectors to target cells. The present invention aims to meet that need.

THE INVENTION

According to this invention, an alternative, novel approach is described for targeting the ecotropic receptor to a specific cell type, and then using unmodified ecotropic vectors to deliver genes to those cells. This has the considerable advantage that the envelope proteins go through the same binding and fusion events as they do when infecting a murine cell.

The invention provides in one aspect an adapter molecule comprising a first amino acid sequence having binding affinity for a cell surface molecule and a second amino acid sequence having binding affinity for a viral surface molecule, which adapter molecule is capable of mediating infection of a cell expressing the cell surface molecule by a viral particle having the viral surface molecule.

Preferably, the second amino acid sequence is derived from a viral receptor, and most preferably it consists essentially of the extracellular portion of the viral receptor to which the viral particle binds.

The first amino acid sequence in the adapter molecule according to the invention may be any amino acid sequence that has a specific affinity for a cell surface molecule. For example, it could be an antibody or antibody derivative, a natural ligand or a derivative of a natural ligand, or a peptide selected by any of various known affinity selection systems such as phage display. An example of an antibody derivative suitable for use in the adapter molecule is a single chain antibody (scFv). An scFv could be used for targeting any specific cell surface molecule. Particularly useful scFv's would be those having binding affinity to tumour-specific cell surface antigens and tissue specific cell surface proteins.

It will be understood that the first and second amino acid sequences as described herein may have the usual modifications associated with proteins and peptides, such as glycosylation.

It will also be understood that in an adapter molecule according to the invention, the first amino acid sequence may be at the carboxy terminus and the second amino acid sequence at the amino terminus, or vice versa.

An adapter molecule according to the invention is capable of mediating infection of a cell by a viral particle as described. The word "infection" as used here means attachment of the viral particle to the cell surface and introduction of genetic material from the viral particle into the

cell. The adapter molecule may thus aid the process of transduction i.e. virus mediated gene transfer.

The first and second amino acid sequences are linked in the adapter molecule either covalently or non-covalently. If covalently linked, the adapter molecule could be produced from a single fusion gene or by chemical cross-linking. If the association is non-covalent the components may be attached by streptavidin-biotin bridges or by means of other binding partners.

In another aspect, the invention provides a nucleic acid coding for an adapter molecule as described herein. Depending on whether intracellular or extracellular expression of the adapter molecule is desired, the nucleic acid may or may not also contain a secretion signal coding region. Where present, the secretion signal coding region could be for example a yeast or an *E.coli* or a mammalian signal for expression and secretion in yeast or *E.coli* or mammalian cells.

The viral surface molecule to which the adapter molecule according to the invention binds is preferably but not necessarily an unmodified ecotropic viral protein. The invention thus permits targeting of viral vectors to specific cell surface molecules with a minimum of modification to the viral envelope. However, it may be necessary or advantageous to modify part of the viral envelope e.g. the particular viral surface molecule involved.

In a further aspect the invention provides genetically engineered cells containing nucleic acids as herein described and capable of expressing adapter molecules as herein described.

In the context of gene therapy, adapter molecules described herein would be administered to the target cells either *in vivo* or *in vitro*, just prior to the administration of corresponding ecotropic vector particles. One aspect of the invention also provides the use of an adapter molecule

as described herein in the manufacture of a medicament for use in gene therapy.

In the attached figures:

Figure 1 is a schematic illustration of a basic amino acid transporter in a membrane.

Figure 2 shows the principle of the targeting molecular adapters according to the invention.

Figure 3 shows a scheme for constructing one embodiment of a targeting adapter according to the invention.

The receptor for all murine ecotropic viruses (F-MLV, R-MLV and Mo-MLV) is the murine cationic amino acid transporter (Albritten et al., 1989 Cell 57, 659; Kim et al., 1991 Nature 352, 725; Wang et al., 1991 Nature 352, 729). It will be referred to here at the Ecotropic Retrovirus Receptor (ERR). This is an integral membrane protein related to the yeast transporters for arginine, histidine and choline. By analogy with other transporters and by using membrane protein prediction programmes putative membrane spanning regions can be identified in the amino acid sequence of the transporter. These are shown schematically in Figure 1. Using the MEMSAT programme (Jones et al., 1994 Biochemistry 33, 3038) we have predicted the coordinates of the transmembrane regions to be 37-58, 65-84, 108-129, 166-183,192-210, 243-266, 281-305, 330-349, 379-396, 404-423, 487-510, 519-540, 553-572 and 582-599.

Several studies, including the analysis of specific mutants and comparisons of human and murine cationic amino acid transporters, suggest that the virus interacts with the 3rd extracellular domain (210-243) of the protein. This has the amino acid sequence:

VKGSIKNWQLTEEDFGCNNNDTNVKYGEGGFMP [SEQ ID NO: 1]

The tyrosine and glutamate residues shown in bold have been specifically implicated in virus infection (Eiden et al., 1993 J.Virol. 67, 4056; Yoshimoto et al., 1993 J.Virol. 67, 1310; Kavanaugh et al., 1994 JBC 269, 15445;

Yoshimoto et al., 1993 J.Virol.67, 1310; MacLeod et al.,1990 MCB 10, 3663). This region of the protein will be referred to here as ERRed3.

In one particular embodiment of the invention ERRed3 is linked to a second molecule that targets it to a specific cell surface molecule. The combination molecule is known as a targeting adapter. This would achieve, for example, the specific targeting of the third domain to a specific cell type. Once attached to the specific cell type the 3rd domain is used as a receptor for ecotropic retroviral vector particles. This would achieve the selective delivery of genes carried by the retroviral vector to specific cell types. The system would be of general use in gene therapy.

EXAMPLES

EXAMPLE 1

In this first example a single chain antibody (scFv) directed against the cell surface marker CD4 is fused to ERRed3 in order to target the receptor fragment to CD4+ cells. This adapter molecule is useful in delivering an anti-HIV gene to HIV susceptible cells.

The technology for making scFv's is well established and generally available through kits supplied by commercial organisations such as Pharmacia Biotech and Novagen. An scFv coding fragment is produced from RNA from a hybridoma expressing the OKT4 monoclonal antibody using the Pharmacia Biotech kit. This fragment is then linked, using standard genetic manipulation techniques, to a coding sequence for ERRed3. This latter fragment is produced by PCR using the following primers:

ACC GCG GCC GCA UGC GTA GGC TCC ATT AAA AAC TGG [SEQ ID NO: 2]

ACC GCG GCC GCA GGG CAT AAA CCC TCC CTC ACC [SEQ ID NO:3]

The fragment encodes amino acids 210-243 with an additional cysteine residue on each end to provide a constrained loop analogous to the authentic extracellular domain.

The structure of the fusion gene would be as shown in Figure 3a (ERR-3 = 3rd extracellular region of the ecotropic retrovirus receptor; sites in parentheses are sites destroyed by ligation). A short coding sequence encoding a secretion signal sequence is then added to the 5' end of this coding sequence to produce the coding sequence shown in Figure 3b. The secretion signal coding sequence might be from any gene such as tPA or immunoglobulin genes (tPAss = tPA secretion signal sequence). In this example the tPA sequence is used and this is added by ligating an oligonucleotide of the sequence:

ATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG CTG CTG CTG TGT

AGC

GGA GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT GCC CGA TTC AGA

AGA GGA GCC AGA TCT TAC TCG [SEQ ID NO: 4]

This will produce a fragment (Figure 3b) that encodes an adapter, designated OKT4sc-ERRed3 that is secreted from mammalian cells. This fragment is then inserted into a mammalian expression vector such as pCI-neo (Promega) and the resulting plasmid designated pCIn-ADAPT-OKT4. This plasmid is then used to transiently transfect 293T cells. 48 hours later the cell supernatant is harvested. This supernatant contains the secreted adapter.

The supernatant sample is filter sterilised and then 1 ml is placed in a dish with HeLa-CD4 cells. In a control experiment 1 ml of supernatant from untransfect d 293T cells is also placed in a dish with HeLa-CD4 cells. The cells are left exposed to the supernatants for 1 hour and then 1ml of ecotropic retroviral vector preparation containing 10^6

transducing particles/ml are added to both dishes. The retroviral vector particles are prepared using the HIT vector system (Soneoka et al., 1995 NAR 23, 628) and they carry the vector genome HIT111 which contains the *E.coli* lacZ gene. After 48 hours the dishes are stained with X-gal and blue cells that have received the vector are counted. In the absence of supernatant containing the adapter no blue cells are seen. With the adapter approximately 10^4-10^5 transductants/ml of vector are observed. This shows that the adapter is mediating gene transfer to CD4+ cells. In further experiments no blue cells are seen in with the adapter supernatant when HeLa cells are used and there is a substantial reduction in blue cells when OKT4 antibody is added to the HeLa-CD4 cells prior to treatment with the adapter supernatant.

The adapter molecules could be produced by a variety of gene fusions that are subtly different at the junctions between the scFv and ERRed3.

EXAMPLE 2

A similar strategy to that described in Example 1 can be used except that the targeting molecule is not an antibody but any other ligand that binds to a cell surface molecule. In this example the first three extracellular immunoglobulin domains of VCAM are fused to ERRed3 using standard recombinant DNA procedures. The VCAM-ERRed3 protein is made in mammalian, yeast or *E.coli* expression systems and then used, as described above for the OKT4svERRed3 protein, to target retroviral vectors to VLA4-bearing cells.

EXAMPLE 3

Constructions as described in Examples 1 and 2 but where the ERRed3 sequence is the amino terminal component and the OKT4 scFv or the VCAM1 domain is the carboxy terminal component.

CLAIMS

1. An adapter molecule comprising a first amino acid sequence having binding affinity for a cell surface molecule and a second amino acid sequence having binding affinity for a viral surface molecule, which adapter molecule is capable of mediating infection of a cell expressing the cell surface molecule by a viral particle having the viral surface molecule.

2. An adapter molecule as claimed in claim 1, wherein the second amino acid sequence is derived from a viral receptor.

3. An adapter molecule as claimed in claim 2, wherein the second amino acid sequence consists essentially of the extracellular portion of the viral receptor to which the viral particle binds.

4. An adapter molecule as claimed in any one of claims 1 to 3, wherein the first amino acid sequence is a single chain antibody directed against the cell surface molecule.

5. An adapter molecule as claimed in any one of claims 1 to 3, wherein the first amino acid sequence is derived from a natural binding ligand for the cell surface molecule.

6. An adapter molecule as claimed in any one of claims 1 to 5, wherein the first and second amino acid sequences are fused by means of covalent bonds

7. An adapter molecule as claimed in any one of claims 1 to 6, for use in gene therapy.

8. A nucleic acid coding for an adapter molecule as claimed in any one of claims 1 to 7.

9. A nucleic acid as claimed in claim 8 which also encodes a secretion signal.

10. A genetically engineered cell containing a nucleic acid as claimed in claim 8 or claim 9, which cell is capable of expressing an adapt r molecule as claimed in any one of claims 1 to 6.

11. The use of an adapter molecule as claimed in any one of claims 1 to 7, in the manufacture of a medicament for use in gene therapy.

Figure 1

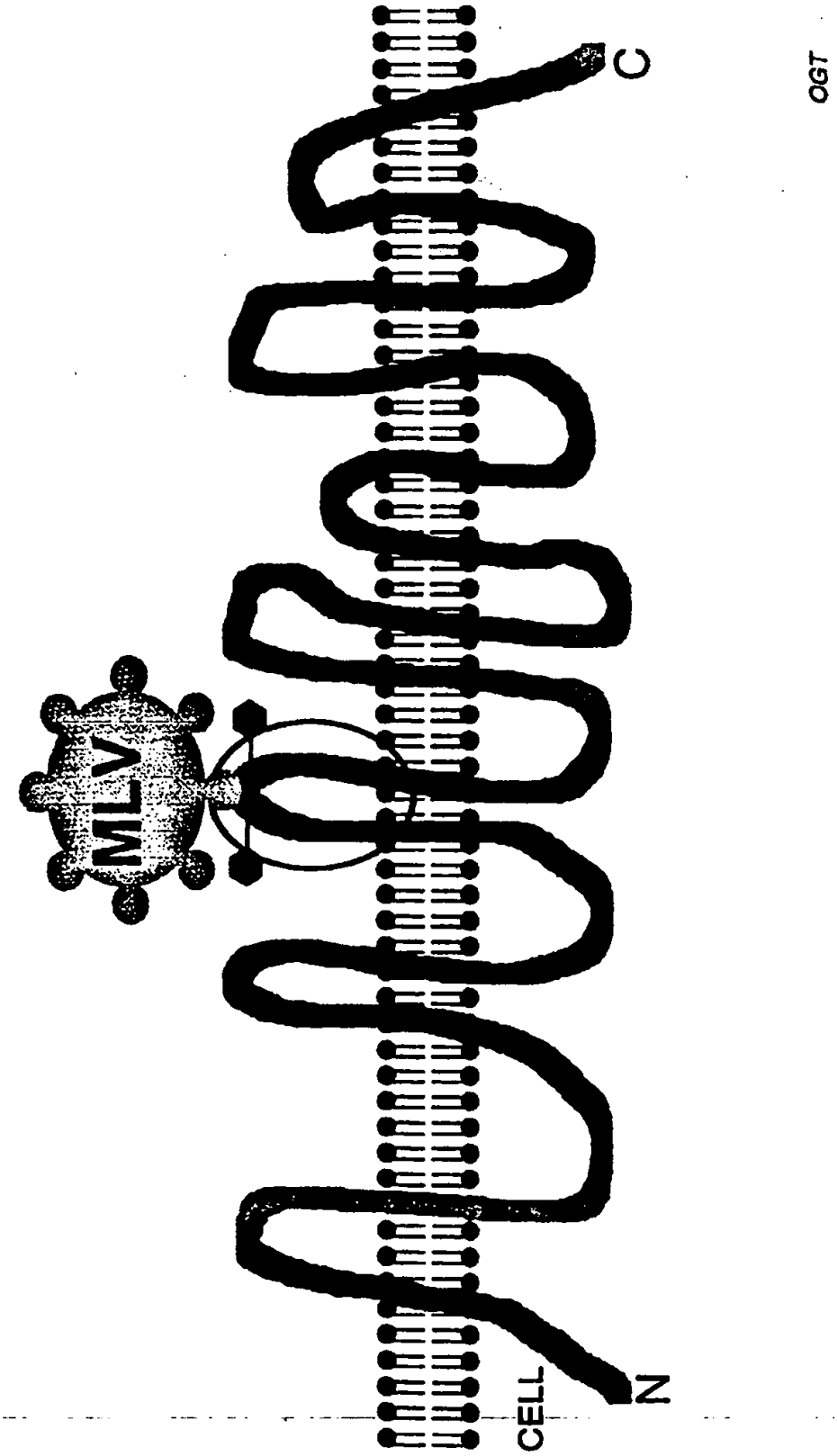


Figure 2

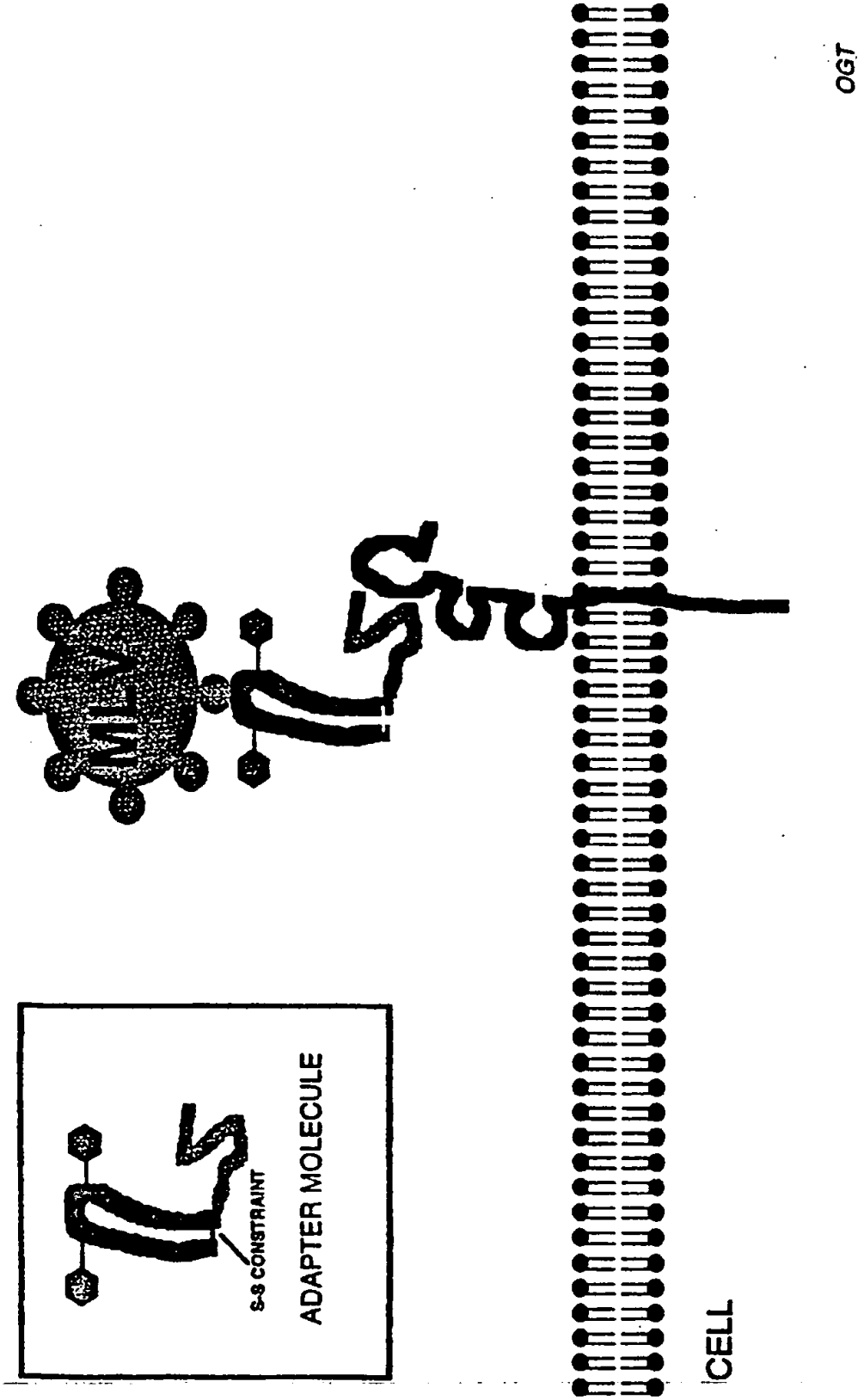
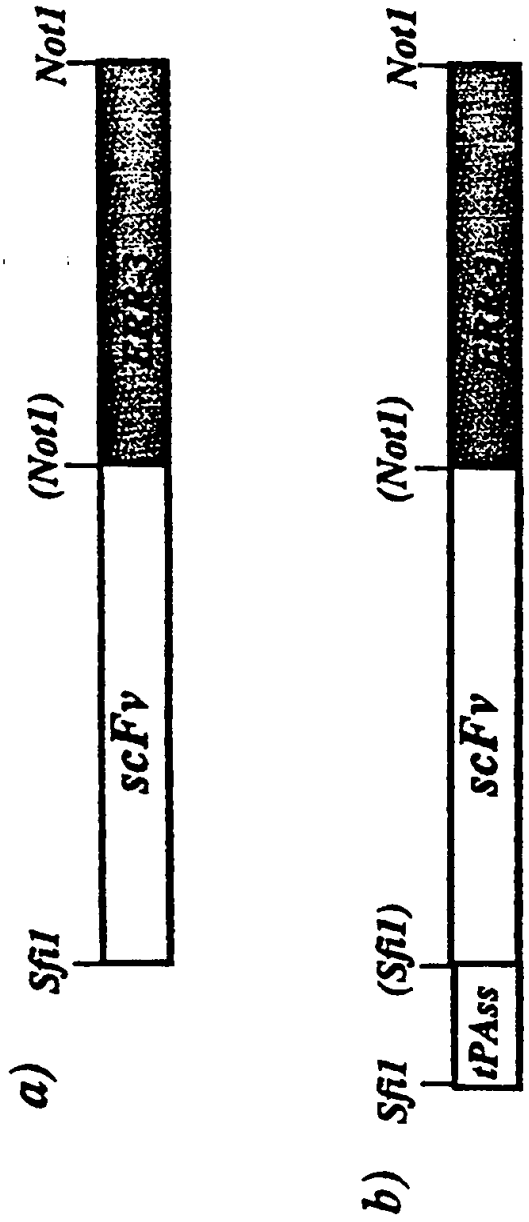


Figure 3



INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C07K19/00 A61K48/00 C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 86, no. 23, 1 December 1989, pages 9079-9083, XP000081882 ROUX P ET AL: "A VERSATILE AND POTENTIALLY GENERAL APPROACH TO THE TARGETING OF SPECIFIC CELL TYPES BY RETROVIRUSES: APPLICATION TO THE INFECTION OF HUMAN CELLS BY MEANS OF MAJOR HISTOCOMPATIBILITY COMPLEX MURINE LEUKEMIA VIRUS-DERIVED VIRUSES" see the whole document ---	1-11
Y	WO 95 23846 A (UNIV MEDICINE & DENTISTRY OF N) 8 September 1995 see the whole document ---	1-11
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CELL, vol. 57, 1989, pages 659-666, XP002036212 ALBRITTON ET AL.: "A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection" cited in the application see the whole document	1-11
A	FASEB JOURNAL, vol. 9, no. 2, February 1995, pages 190-199, XP000616414 MILLER N ET AL: "TARGETED VECTORS FOR GENE THERAPY" see page 192, right column see the whole document	1-11
A	GENE THERAPY, vol. 1, no. suppl.2, 1994, pages S15-A59, XP002036213 MARIN ET AL.: "Cell targeting by murine recombinant retroviruses" see the whole document	1-11
A	GENE THERAPY, vol. 1, no. 5, September 1994, pages 292-299, XP000646936 TE-HUA TEARINA CHU ET AL: "CELL TARGETING WITH RETROVIRAL VECTOR PARTICLES CONTAINING ANTIBODY-ENVELOPE FUSION PROTEINS" see the whole document	1-11
A	SCIENCE, vol. 266, no. 5189, 25 November 1994, pages 1373-1376, XP000541772 KASAHARA N ET AL: "TISSUE-SPECIFIC TARGETING OF RETROVIRAL VECTORS THROUGH LIGAND-RECEPTOR INTERACTIONS" see the whole document	1-11

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9523846 A	08-09-95	CA 2184354 A	08-09-95